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14. ABSTRACT The first significant achievement during this period includes strongly encouraging results of cellular studies with prostate cancer cells to demonstrate that a combination of 1,25-dihydroxyvitamin D3-3-bromoacetate (1,25(OH)2D3-3-BE) and another compound named Largazole synergistically reduced the growth of these cells. Combination therapy in cancer is a well-recognized modality in cancer-treatment. Therefore these results are highly significant in moving our drug (1,25(OH)2D3-3-BE), forward for prostate cancer-treatment. The second achievement involves development of a liposomal preparation of 1,25(OH)2D3-3-BE, and demonstration that this preparation is superior to 'naked' drug both in in vitro and in vivo assays.					
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Introduction

Therapeutic potential of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) in prostate cancer is well-recognized. However, its clinical use has been restricted by its inherent calcemic toxicity. In recent studies we demonstrated that 1 α ,25-dihydroxyvitamin D₃-3-bromoacetate [1,25(OH)₂D₃-3-BE], a derivative of 1,25(OH)₂D₃ that covalently links 1,25(OH)₂D₃ inside the ligand-binding pocket of nuclear vitamin D receptor (VDR) is a strong antiproliferative and pro-apoptotic agent in several androgen-sensitive and androgen-refractory human prostate cancer cells. Furthermore, 1,25(OH)₂D₃-3-BE demonstrated strong anti-prostate tumor effect in athymic mice without toxicity. The goal of this project is to evaluate the translational potential of 1,25(OH)₂D₃-3-BE as a therapeutic agent for prostate cancer. This will be achieved by determining the efficacy of 1,25(OH)₂D₃-3-BE in mouse models of human androgen-sensitive and androgen-insensitive prostate cancer, as well as evaluating its molecular mechanisms of action in several *in vitro* studies.

Studies completed/goals achieved during the one year period (Year 4) of the project

I. Continuation of our trials to develop androgen-sensitive LNCaP tumors in athymic mice

As described in our previous report (#3) we have completed an efficacy study of our compound (1,25(OH)₂D₃-3-BE) in a mouse xenograft model of androgen-insensitive (DU-145) prostate cancer, and demonstrated a strong translational potential of this compound in androgen-insensitive prostate cancer. However, all our attempts to develop androgen-sensitive tumor in nude mice (with LNCaP cells) have been unsuccessful. We have communicated with several researchers who have published papers on this tumor, and received several hints to successfully develop this tumor (to study the efficacy of our compound). Currently we are incorporating these hints in our protocol to develop LNCaP and/or LAPC-4 (another androgen-sensitive prostate cancer cell) tumor in nude mice and study the efficacy of 1,25(OH)₂D₃-3-BE.

II. Preparation of a liposomal formulation of 1,25(OH)₂D₃-3-BE, and its *in vitro* and *in vivo* studies in prostate cancer cells and androgen-insensitive prostate cancer mouse xenograft model

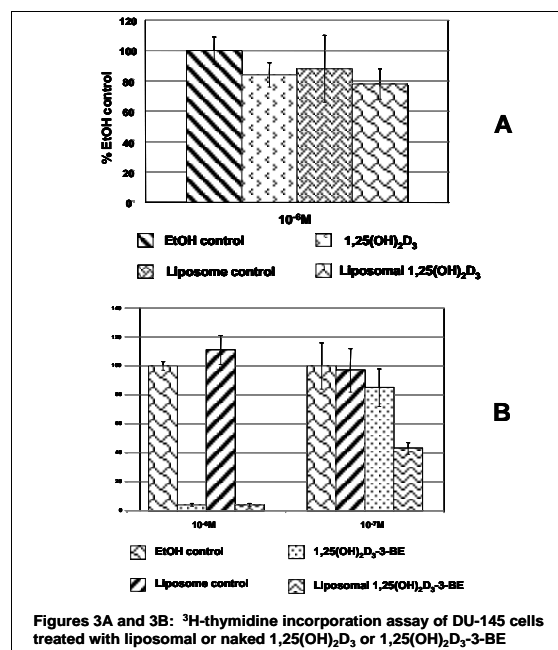
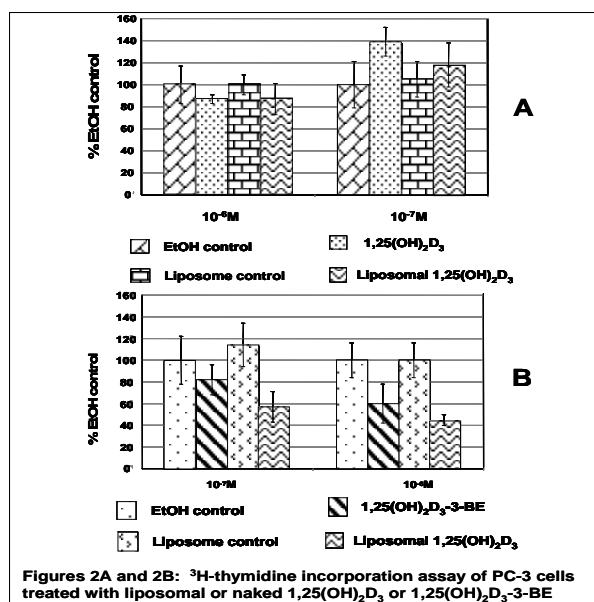
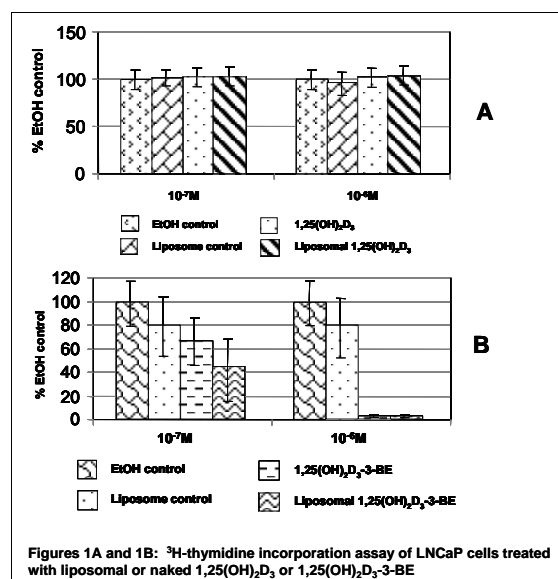
Liposome/nanosomes of drugs and other small molecules are known to protect these molecules from premature degradation as well as targeting these molecules specifically to tumor cells thus reducing toxicity. Our results in androgen-insensitive prostate cancer tumor model (Report #3) strongly suggest a translational potential of our compound *i.e.* 1,25(OH)₂D₃-3-BE. Therefore, we prepared a liposomal formulation of 1,25(OH)₂D₃-3-BE and studied its *in vitro* and *in vivo* properties, as delineated below.

Preparation of liposomes: A solution of cholesterol (1 μ g), dimethylphosphatidyl choline (DMPC) (20 μ g) and 1,25(OH)₂D₃ (1 μ g) or 1,25(OH)₂D₃-3-BE ((1 μ g) in chloroform was dried in a stream of argon. Phosphated saline (PBS, 2.5 ml) was added to the solid residue followed by mixing by brief vortexing and the mixture was sonicated for 15 min. The milky solution was incubated at 50°C for 50 min and frozen at -77°C for 20 min. This heating and freezing cycle was repeated once, and the preparation was stored at 4°C for use in assays. Prior to each assay the liposomal preparation was sonicated and vortexed briefly for proper mixing.

Antiproliferative assays: We tested antiproliferative activity of 1,25(OH)₂D₃, liposomal 1,25(OH)₂D₃ [(1,25(OH)₂D₃)_L], 1,25(OH)₂D₃-3-BE and liposomal 1,25(OH)₂D₃-3-BE [(1,25(OH)₂D₃-3-BE)_L] in three (3) prostate cancer cells (LNCaP, PC-3 and DU-145 cells) by ³H-thymidine incorporation or MTT assays.

³H-thymidine-incorporation assay: In a typical assay cells were grown to 50-60% confluence in 24-well plates in respective media containing 5% FBS, serum-starved for 20 hours, followed by treatment with various concentrations of 1,25(OH)₂D₃ or 1,25(OH)₂D₃-3-BE (in 0.1% ethanolic solution) or ethanol (vehicle) or (1,25(OH)₂D₃)_L or (1,25(OH)₂D₃-3-BE)_L or blank liposome in serum-containing medium for 16 hours. After the treatment media was removed from the wells and replaced with media containing ³H-thymidine (Sigma, 0.1μCi) per well, and the cells were incubated for 3 hours at 37°C. After this period media was removed by aspiration and the cells were washed thoroughly (3 X 0.5 ml) with PBS. Then ice-cold 5% perchloric acid solution (0.5 ml) was added to each well and the cells were incubated on ice for 20 minutes. After this incubation, perchloric acid was removed by aspiration, replaced with 0.5 ml of fresh perchloric acid solution and the cells were incubated at 70°C for 20 minutes. Solution from each well was mixed with scintillation fluid and counted in a liquid scintillation counter. There were six (6) wells per sample, and statistics was carried out by Student's t test.

MTT assay: Growth-inhibitory activity of 1,25(OH)₂D₃-3-BE vs. (1,25(OH)₂D₃-3-BE)_L in

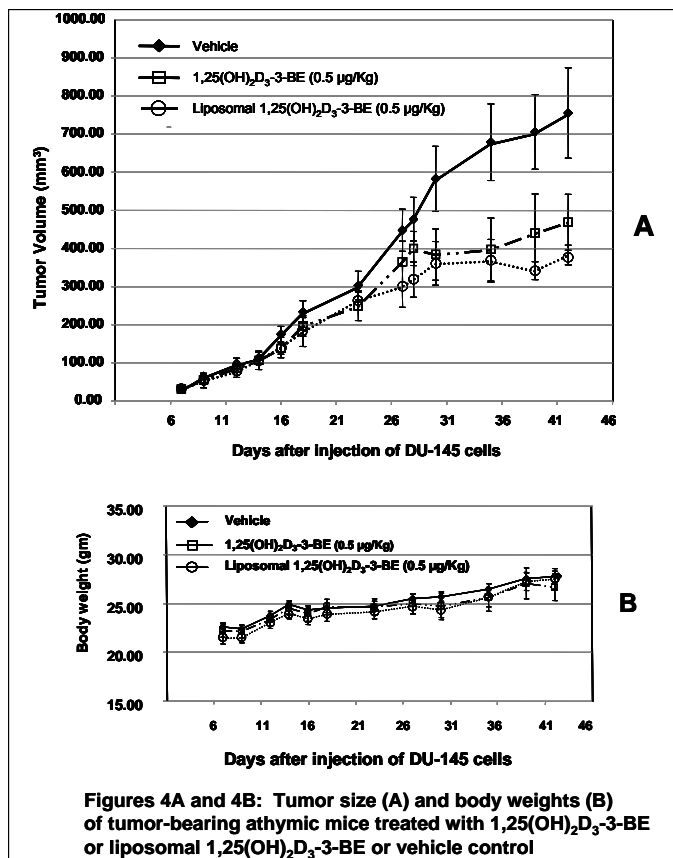


LNCaP cells was determined by MTT assay according to manufacturer's procedure (Trevigen, Gaithersburg, MD). In this assay yellow 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is reduced to purple crystals in the mitochondria of living cells which is dissolved in DMSO and measured spectrophotometrically.

Results, shown in Figures 1-3 strongly indicate that liposomal formulation of 1,25(OH)₂D₃-3-BE has a strong and dose-dependent antiproliferative effect on the prostate cancer cells.

But, $1,25(\text{OH})_2\text{D}_3$ under the same conditions, showed practically no effect.

***In vivo* efficacy studies of $1,25(\text{OH})_2\text{D}_3$ -3-BE, and liposomal $1,25(\text{OH})_2\text{D}_3$ -3-BE in athymic mice inoculated with DU-145 human prostate cancer cells:**



Male, athymic mice (Taconic, NY, average weight 20 gm) were fed normal rat chow and water *ad libitum*. They were inoculated with DU 145 cells, grown in culture in the flank under light anesthesia. When the tumor size grew to approximately 100 mm³ the animals were randomized into groups of ten (10) tumor-bearing animals, and they were given $1,25(\text{OH})_2\text{D}_3$ -3-BE (0.5 µg/kg) or liposomal $1,25(\text{OH})_2\text{D}_3$ -3-BE (0.5 µg/kg) or vehicle (5% dimethylacetamide, DMA in sesame oil) by either intraperitoneal injection (*i.p.*) on approximately every third day. Treatment started on day 7 and stopped on day 42. Body-weights of the animals were measured on the days of administration of various agents.

Results of this experiment are shown in **Figure 4A and B**. At the end of the experiment average size of vehicle-control, $1,25(\text{OH})_2\text{D}_3$ -3-BE-treated and

$(1,25(\text{OH})_2\text{D}_3\text{-3-BE})_L$ -treated tumors were approximately 750, 475 and 385 mm³ respectively (**Figure 4A**), demonstrating a strong reduction of tumor size by $1,25(\text{OH})_2\text{D}_3$ -3-BE (63% of control) and $(1,25(\text{OH})_2\text{D}_3\text{-3-BE})_L$ (51% of control). On the other hand, gross body-weights of $1,25(\text{OH})_2\text{D}_3$ -3-BE- and $(1,25(\text{OH})_2\text{D}_3\text{-3-BE})_L$ -treated animals were not significantly different from control animals, indicating lack of toxicity (**Figure 4B**). Therefore, collectively these results demonstrated that $1,25(\text{OH})_2\text{D}_3$ -3-BE, particularly $(1,25(\text{OH})_2\text{D}_3\text{-3-BE})_L$ has a strong translational potential as a therapeutic agent in androgen-insensitive prostate cancer.

KEY RESEARCH ACCOMPLISHMENTS

Development of a liposomal formulation of $1,25(\text{OH})_2\text{D}_3$ -3-BE and demonstration that it has a better antitumor effect than the naked variety.

REPORTABLE OUTCOME

During the past one year our efforts with $1,25(\text{OH})_2\text{D}_3$ -3-BE and a related studies has generated one publication, one (1) meeting abstract and an invited lecture.

CONCLUSION

Our effort for the past one year has strongly endorsed the translational potential of 1,25(OH)₂D₃-3-BE in prostate cancer. Currently we are engaged in determining the effect of 1,25(OH)₂D₃-3-BE in androgen-sensitive prostate tumor model.

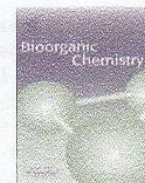
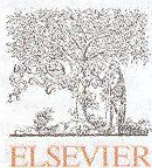
APPENDIX

Meeting Abstracts:

Experimental Biology, 2009, New Orleans, LA, April 18-April 22, 2009

Dietary vitamin D and calcium intake on prostate tumor progression in athymic mice: high calcium intake does not enhance prostate tumor growth. Rahul Ray, Hilal Abuzahra, Andrew Tannenbaum, Michael Holick.

Invited Lecture: Vitamin D and cancer. Chittaranjan National Cancer Institute, Kolkata, India, July 8, 2009



Covalent labeling of nuclear vitamin D receptor with affinity labeling reagents containing a cross-linking probe at three different positions of the parent ligand: Structural and biochemical implications

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Structural elements in vitamin D receptor–ligand-binding domain
Molecular modeling

ABSTRACT

Structure–functional characterization of vitamin D receptor (VDR) requires identification of structurally distinct areas of VDR–ligand-binding domain (VDR-LBD) important for biological properties of 1 α ,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃). We hypothesized that covalent attachment of the ligand into VDR-LBD might alter ‘surface structure’ of that area influencing biological activity of the ligand. We compared anti-proliferative activity of three affinity alkylating derivatives of 1,25(OH)₂D₃ containing an alkylating probe at 1,3 and 11 positions. These compounds possessed high-affinity binding for VDR; and affinity labeled VDR-LBD. But, only the analog with probe at 3-position significantly altered growth in keratinocytes, compared with 1,25(OH)₂D₃. Molecular models of these analogs, docked inside VDR-LBD tentatively identified Ser237 (helix-3: 1,25(OH)₂D₃-1-BE), Cys288 (β -hairpin region: 1,25(OH)₂D₃-3-BE,) and Tyr295 (helix-6: 1,25(OH)₂D₃-11-BE,) as amino acids that are potentially modified by these reagents. Therefore, we conclude that the β -hairpin region (modified by 1,25(OH)₂D₃-3-BE) is most important for growth inhibition by 1,25(OH)₂D₃, while helices 3 and 6 are less important for such activity.

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1. Introduction

1 α ,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃), the dihydroxylated metabolite of vitamin D₃ serves multiple functions. Its biological properties include calcium and phosphorus-homeostasis, growth and maturation-control of a broad range of malignant cells, and immune-regulation. As a result the therapeutic potential of 1,25(OH)₂D₃ in a broad range of diseases, including mineral homeostatic diseases such as renal osteodystrophy, proliferative diseases such as psoriasis and cancer, and immune-deficiency diseases, such as type I diabetes mellitus is well-recognized [1–7]. However, inherent toxicity of

the parent hormone (hypercalcemia, hypercalciuria), particularly at pharmacological doses, has largely precluded its general use as a therapeutic agent. This limitation has spawned a strong interest in developing analogs of 1,25(OH)₂D₃ that retain intact beneficial effects but display reduced toxicity. Several such analogs have shown promise, but have displayed only a moderate effect clinically in non-toxic doses [8–12]. It is amply clear that rational development of such analogs will require proper understanding of their mechanism of action at the molecular level.

According to current dogma, 1,25(OH)₂D₃ binds to its nuclear receptor, vitamin D receptor (VDR) in target cells with high specificity; allosterically promoting heterodimerization with the retinoid X receptor (RXR), and binding of the VDR–1,25(OH)₂D₃–RXR complex to vitamin D response elements (VDREs) in the vitamin D-regulated genes [e.g., osteopontin, osteocalcin, 1 α ,25-dihydroxyvitamin D₃-24-hydroxylase (CYP-24)] and recruitment of co-activators to initiate transcription and translation [13,14]. In an alternative proposal apo-VDR, bound to co-repressors, remains transcriptionally inactive till 1,25(OH)₂D₃ binds to initiate the multi-step transcriptional process. The most important among all the steps in this transcriptional process is highly specific interaction between 1,25(OH)₂D₃ and VDR. Therefore, structure–functional knowledge, from the sides of both VDR and 1,25(OH)₂D₃ is

Abbreviations: 1,25(OH)₂D₃, 1 α ,25-dihydroxyvitamin D₃; VDR, nuclear vitamin D receptor; reVDR, recombinant vitamin D receptor; VDR-LBD, vitamin D receptor–ligand-binding domain; 1,25(OH)₂D₃-1-BE, 1 α ,25-dihydroxyvitamin D₃-1 α -(2)-bromoacetate; ³H-1,25(OH)₂D₃-1-BE, 1 α ,25-dihydroxy[26(27)-³H]vitamin D₃-1 α -(2)-bromoacetate; 1,25(OH)₂D₃-3-BE, 1 α ,25-dihydroxyvitamin D₃-3 β -(2)-bromoacetate; ¹⁴C-1,25(OH)₂D₃-3-BE, 1 α ,25-dihydroxyvitamin D₃-3 β -[1-¹⁴C]-(2)-bromoacetate; 1,25(OH)₂D₃-6-BE, 1 α ,25-dihydroxyvitamin D₃-6-propoxy-(2)-bromoacetate; 1,25(OH)₂D₃-11-BE, 1 α ,25-dihydroxyvitamin D₃-11 α -hydroxy-(2)-bromoacetate; ¹⁴C-1,25(OH)₂D₃-11-BE, 1 α ,25-dihydroxyvitamin D₃-11 α -hydroxy-[1-¹⁴C]-(2)-bromoacetate.

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